Chapter 7
Glutathione-Related Enzyme System: Glutathione Reductase (GR), Glutathione Transferases (GSTs) and Glutathione Peroxidases (GPXs)

J. Csiszár, E. Horváth, K. Bela and Á. Gallé

Abstract The glutathione-related enzymes are usually considered to accompany the main non-enzymatic antioxidative compounds of the ascorbate–glutathione cycle. Taking into account that the redox processes are not spontaneous in cells, but the adequate reaction velocity and appropriate specificity are achieved by the catalyzing activity of enzymes, special attention has raised toward the glutathione-utilizing enzymes. Glutathione reductase (GR) is a NADPH-dependent oxidoreductase which catalyzes the conversion of oxidized glutathione (GSSG) to reduced glutathione (GSH). Some members of the diverse glutathione transferase (GST) enzyme family have GSH-dependent thiol transferase activity and participate in the recycling of antioxidants (ascorbate, flavonoids, quinones), while other isoenzymes, due to their *S*-transferase activity, are involved in the detoxification mechanisms using GSH as co-substrate. A significant portion of GST isoenzymes also has glutathione peroxidase activity and can convert lipid peroxides and other peroxides to less harmful compounds. The plant glutathione peroxidase enzymes (GPXs) may be involved in the detoxification of H_2O_2 and organic hydroperoxides and in the regulation of the cellular redox homeostasis by maintaining the thiol/disulfide balance. Most of plant GPXs prefer to use thioredoxin (TRX) instead of glutathione as a reducing agent, and it is thought that the GPXs may represent a link between the glutathione- and the thioredoxin-based system. The GR, GPX and some GST isoenzymes have Cys in their active center and thus are directly regulated by redox status. This chapter summarizes their roles in stress responses as antioxidant enzymes, in determining the redox status of cells, and emphasizes their connection to redox signaling mechanisms.

Keywords Antioxidant enzymes · Glutathione · Reactive oxygen species · Redox homeostasis · Redox regulation

J. Csiszár (✉) · E. Horváth · K. Bela · Á. Gallé
Department of Plant Biology, University of Szeged, Szeged Közép fasor 52, 6726, Hungary
e-mail: csiszar@bio.u-szeged.hu

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7.1 Introduction

Cellular redox homeostasis is an essential buffering mechanism that prevents excessive reduction or oxidation. There is a close interplay among the individual redox-active molecules, and the status of each of them can influence the plant metabolism and environmental responses. Key participants in the redox processes are the ascorbate and glutathione which are central components of the highly complex plant antioxidative system (Foyer and Noctor 2011). Their common features are that (i) they are abundant and are present in plants in mmolar (0.5–10 mM) concentrations; (ii) specific enzymes couple them to peroxide metabolism; (iii) their oxidized forms are relatively stable; and (iv) the recycling of these forms to the reduced compounds is done by high-capacity enzyme-based systems that depend on NAD(P)H, the key electron carriers (Noctor and Foyer 1998). It was suggested that NAD(P)H serves best in the organization and the control over energy production pathways, ascorbate is the redox molecule which primarily regulates development, and glutathione is involved in plant development, but is mainly important for stress defense and signaling (Potters et al. 2010). The aim of this chapter is to provide an overview of the cellular functions of glutathione in relation to the determination of the redox homeostasis. The redox processes are usually not spontaneous in the cells, but the adequate reaction velocity and appropriate specificity are achieved by the catalyzing activity of enzymes, so special attention has risen toward the GSH-utilizing enzymes. They are usually considered to accompany the main non-enzymatic antioxidative compounds of the ascorbate–glutathione cycle. Besides summarizing their roles in stress responses as antioxidant enzymes, this chapter deals with their functions in determining the redox status of cells and emphasizes the latest results and suggested roles as a key participant in redox signaling mechanisms.

7.2 Glutathione

All plants contain the tripeptide glutathione (γ-Glu-Cys-Gly, GSH) or GSH homologues, where the C-terminal amino acid glycine is replaced by another amino acid, such as β-alanine, serine or glutamate (Noctor et al. 2012). GSH is synthesized by two ATP-dependent steps. First, γ-glutamylcysteine is formed by the plastidic glutamate–cysteine ligase, also known as γ-glutamylcysteine synthetase (γ-ECS or GSH1), which is the rate-limiting reaction. Glutathione synthetase (GSH2) then catalyzes the addition of glycine to γ-glutamylcysteine (Noctor et al. 2012). GSH synthesis takes place in the cytosol and the chloroplasts (Diaz-Vivancos et al. 2015), and in *Brassicaceae*, both enzymes are encoded by single genes with alternate transcription start sites that are associated with their subcellular localization (Wachter et al. 2005). Knockout mutations in *GSH1* prevent embryo development in maturing seeds (Cairns et al. 2006). A number of mutants with defects in

the *GSH1* gene have been identified in *Arabidopsis thaliana*, including *root meristemless1 (rml1)*, *regulator of APX2 1-1* (*rax1-1*), *cadmium-sensitive mutant2-1* (*cad2-1)* and *phytoalexin-deficient2-1* (*pad2-1)*. Knockout mutations in *GSH2* allow seed germination, but seedling development is arrested at an early stage (Pasternak et al. 2008).

GSH is abundant in the plant cell cytosol, chloroplasts, mitochondria, peroxisomes and nucleus (Muller et al. 2004; Zechmann et al. 2008; Diaz-Vivancos et al. 2015). It fulfills a broad range of essential functions including detoxification of heavy metals and xenobiotics and serving as an electron-donating cofactor in biochemical reactions (Cobbett and Goldsbrough 2002; Noctor et al. 2011). When GSH reacts with oxidants, it becomes converted into the oxidized form, glutathione disulfide (GSSG). As a result of the reversible convertibility between the reduced and the oxidized form and the relatively high concentration of the GSH in the cells, glutathione is one of the most important redox buffer systems. Shifts in the cellular glutathione redox state may reversibly modify redox-sensitive thiol groups in target proteins, either through glutathionylation or through formations of cysteine cross-bridges, with specificity conferred by glutaredoxins (GRXs). Many reports indicate that the GSH/GSSG ratio is an effective marker of the cellular redox homeostasis. From the concentrations of GSH and GSSG, the half-cell reduction potential ($E_{GSSG/2GSH}$) can be calculated (Schafer and Buettner 2001). Glutathione redox potential (E_{GSH}) depends on the absolute glutathione concentration and the ratio of [GSH] to [GSSG] (Meyer and Hell 2005). The cytosolic GSH buffer is normally highly reduced with more negative E_{GSH} than −310 mV (Aller et al. 2013). Increase of E_{GSH} to −260 mV (e.g., in the roots of *rml1* mutants) is sufficient to prevent the cell cycle G_1/S transition and causes large changes in the transcript profiles of roots and shoots (Aller et al. 2013; Schnaubelt et al. 2015). The E_{GSH} parameter was found to be correlated with the biological status of the cell and thus could be used as a universal marker of cell viability and to monitor stress-induced damages (Schafer and Buettner 2001; Kranner et al. 2006; Szalai et al. 2009). It was reported that redox changes (H_2O_2 level, ascorbate and GSH concentrations and the ratio of their reduced to oxidized form) and the half-cell reduction potential of the GSH/GSSG couple were correlated with the level of stress tolerance (Soltesz et al. 2011). An overview of the main glutathione-related enzymes and the role of glutathione in plant response to abiotic stresses are shown in Fig. 7.1. As a summary, the glutathione peroxidase (GPX) and dehydroascorbate reductase (DHAR) are involved in converting H_2O_2 into H_2O. The glutathione reductase (GR) regulates the GSH/GSSG ratio and supplies GSH for several enzymes, such as DHAR, GPX, glutathione transferase (GST), phytochelatin synthetase (PCS) and GRXs (Fig. 7.1).

Plants are masters of the art of redox regulation that use oxidants and antioxidants as flexible integrators of signals from metabolism and the environment (Foyer and Noctor 2013). The importance of GSH-related enzymes in the abiotic stress responses was investigated intensively for several decades, but their involvement in the redox processes received special attention only in the recent years.

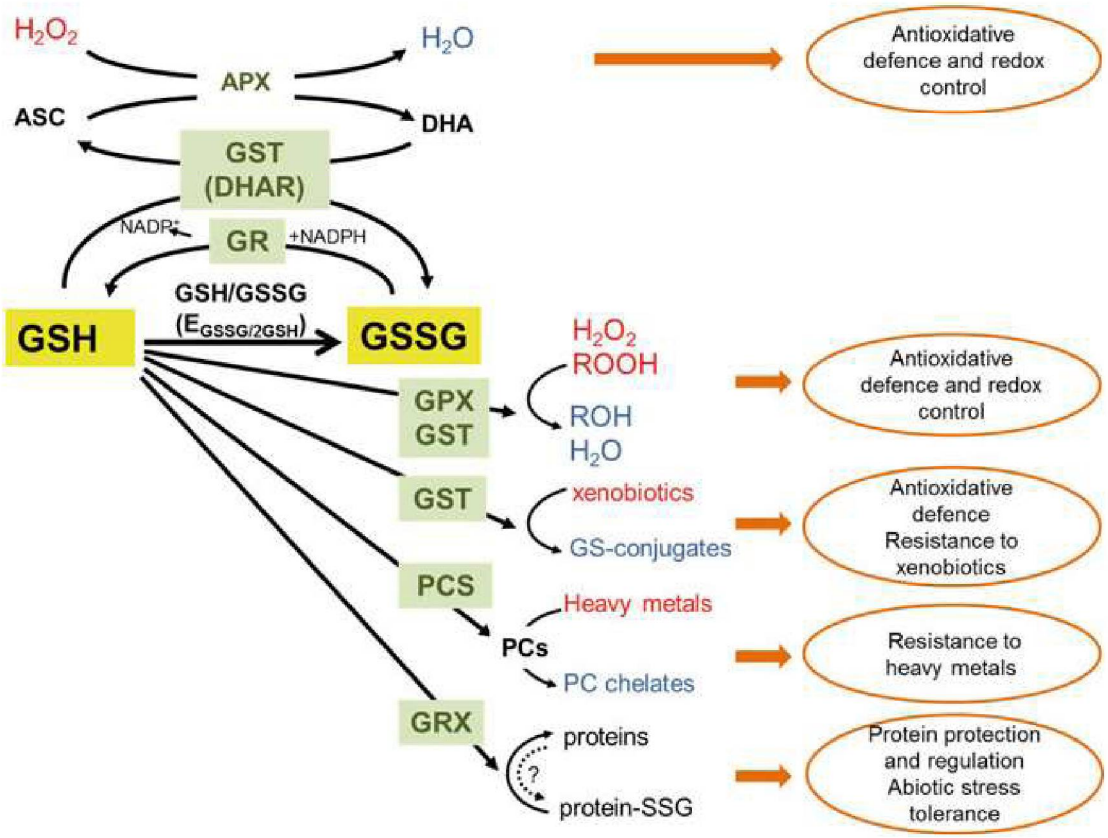

Fig. 7.1 Overview of the role of glutathione in plant response to abiotic stresses. The main glutathione-related enzymes are depicted by *boxes*; their contribution to stress tolerance is highlighted. Potential deleterious compounds and their adducts are also shown. *ASC* ascorbate; *APX* ascorbate peroxidase; *DHA* dehydroascorbic acid; *DHAR* dehydroascorbate reductase; $E_{GSSG/2GSH}$ glutathione half-cell reduction potential; *GR* glutathione reductase; *GPX* glutathione peroxidase; *GRX* glutaredoxin; *GSH* reduced glutathione; *GSSG* oxidized glutathione/glutathione disulfide; *GST* glutathione transferase; *MDHAR* monodehydroascorbate reductase; *PCs* phytochelatins; *PCS* phytochelatin synthetase; *ROH* organic alcohol; ROOH organic hydroperoxide

7.3 Glutathione Reductase Supports Continuous Reduction of the Oxidized Glutathione

High GSH/GSSG ratios are maintained by the activity of glutathione reductase (EC 1.8.1.7), which belongs to the NADPH-dependent oxidoreductase group of flavoproteins and catalyzes the transfer of electrons from NADPH to FAD prosthetic group and ultimately to the reactive disulfide bridge center that reduces GSSG to GSH. GR ensures that the 2GSH/GSSG and $NADP^+$/NADPH redox couples are in thermodynamic equilibrium and hence at the same redox potential (Diaz-Vivancos et al. 2015). Glutathione reductase is ubiquitously found in all organisms from prokaryotes to eukaryotes including plants and mammals (Gill et al. 2013; Trivedi et al. 2013). In plants, GR enzyme was reported first in the 1950s and has subsequently been noted in numerous plant species and tissues (Rennenberg 1982; Rao and Reddy 2008). Recent investigations verified that one of the GR groups

corresponds to the cytosolic and in some cases to the peroxisomal GR, whereas the other accounts for the chloroplastic and mitochondrial GR (Romero-Puertas et al. 2006; Kataya and Reumann 2010; Pang and Wang 2010). Consistent with the early published classifications, the phylogenetic tree of GR proteins constructed by neighbor-joining method revealed that the GR groups of the cytosol and the chloroplast are clustered separately (Zhang et al. 2013). The amino acid sequence identity of GRs in the same cellular organs has a closer genetic relationship than those in the same plants (Rouhier et al. 2006). Cytosolic GRs share higher identity (from 71 to 91 %) than chloroplastic ones (between 64 and 77 %), whereas the homology of the GR sequences from cytosol and chloroplast is only between 41 and 55 % (Contour-Ansel et al. 2006; Rouhier et al. 2006; Pang and Wang 2010).

Although only two classes of plant GRs can be separated according to their protein and cDNA sequences, in higher plants it has been proven that four types of GR occur in the cytosol, chloroplast, mitochondria and peroxisome, respectively (Jimenez et al. 1997; Romero-Puertas et al. 2006; Kataya and Reumann 2010; Yousuf et al. 2012). Peroxisomal GR was purified for the first time from pea leaves, and it was revealed that the molecular properties of this isoform are similar to those described for most GRs (Jimenez et al. 1997; Romero-Puertas et al. 2006). Recently, Delorme-Hinoux et al. (2016) reported that in *Arabidopsis thaliana*, the predominantly cytosolic GR1 protein was also found in the nucleus of leaf and root cells, indicating that GSH reduction is potentially active in this compartment too. GR isoforms with different cellular localization appear to be products of the posttranslational processing mostly of two or sometimes of three genes (Delorme-Hinoux et al. 2016). This difference in the number of genes probably arises from a duplication event. The GR gene structure is well conserved among higher plants, since they are composed usually either of 10/11 exons (chloroplastic) or of 16/17 exons (cytosolic). For instance, in poplar, the gene most likely duplicated is composed of 16 exons, whereas that of rice is the 10 exon variant (Rouhier et al. 2006).

The native enzyme of GR is mostly a homodimer, but it can be also found as monomer and heterodimer in *Chlamydomonas*, maize and pea (Gill et al. 2013; Trivedi et al. 2013). The homodimer form of GR is approximately 100–150 kDa, and its subunit size is between 53 and 59 kDa (Romero-Puertas et al. 2006). GR dimers may be further assembled into tetramers or even higher aggregative states, which are determined by temperature and pH. It was suggested that this may be one of the regulation mechanisms of GR activity (Pang and Wang 2010).

GR contains highly conserved domains in different species for binding flavin adenine dinucleotide (FAD), NADPH and GSSG, and for joining the two subunits in the interface region. The NADPH binding domain consists of altering β sheets and α helixes (βαβα arrangement). The pyridine nucleotide–disulfide oxidoreductases class I active site is evolutionary conserved among the GR proteins, and the GXGXXA motif and Arg residues present therein play an important role in binding the cofactor (Kubo et al. 1993; Rao and Reddy 2008; Trivedi et al. 2013). The active site where GSSG is bound is located between the two subunits. The two Cys of the GR redox center, involved in forming the redox-active disulfide bond, are

separated by four amino acid residues in the highly conserved motif GGTCV[I/L]RGCVPKK[I/L]LVY (Rouhier et al. 2006; Rao and Reddy 2008). The special feature of redox-active Cys residues is that they usually exist as highly reactive thiolate anions ($-S^-$) under physiological pH conditions, because their pKa values, ranging from 3 to 6, are much lower than that of other protein thiols, pKa values of which are greater than 8.0. In contrast to their protonated forms, acidic thiols are highly susceptible to oxidation by reactive oxygen, nitrogen or sulfur species (Spadaro et al. 2010; Couturier et al. 2013; Diaz-Vivancos et al. 2015).

GR plays an essential role in the maintenance of the reduced glutathione pool and has high specificities for its substrates, although it can reduce some glutathione conjugates and also mixed glutathione disulfides (Gaullier et al. 1994; Pang and Wang 2010). One mole of NADPH is required to reduce GSSG to GSH for every mole of GSSG. The catalytic mechanism involves two steps: First, the flavin moiety is reduced by NADPH, the flavin is oxidized, and a redox-active disulfide bridge is reduced to produce a thiolate anion and a Cys. The reduction of GSSG via thiol/disulfide interchange reactions is involved in the second step (Ghisla and Massey 1989; Rao and Reddy 2008; Gill et al. 2013). Analysis of the catalyzed reaction of *Arabidopsis* GR1 revealed Michaelis–Menten kinetics with a K_m for the GSSG substrate of 77 μM and a V_{max} of 63 μmol min^{-1} mg^{-1}. The K_m for the co-substrate NADPH was 33 μM (Marty et al. 2009). In case of *Arabidopsis* chloroplastic GR2, the K_m for GSSG was 44 μM and that for NADPH was 5.0 μM at 25 °C (Kubo et al. 1993). Recently, Achary et al. (2015) reported that the K_m and V_{max} values for GSSG were 153.85 μM and 172.4 μmol min^{-1} mg^{-1} of protein and the K_m for NADPH was 144.93 μM in *Pennisetum glaucum*, respectively. These K_m values can be compared with that of previously reported GR in other organisms that ranged from 12.3 to 114 μM for GSSG and 3.7–56 μM for NADPH (Turner and Pollock 1993; Hakam and Simon 2000; Achary et al. 2015).

Numerous reviews have covered the role of GRs in abiotic stress responses (Rao and Reddy 2008; Noctor et al. 2011; Yousuf et al. 2012), but their function related to the redox state was emphasized only in the recent years. Gill et al. (2013) described that increased GR activity confers stress tolerance and has the ability to alter the redox state of important components of the electron transport chain. With exhaustive literature search, they have revealed differential modulation of GR activity in different plant species exposed to metal–metalloids, salinity, drought and various abiotic stresses. Under stress conditions, GR plays a central role in maintaining the reduced glutathione pool in the cellular environment and strengthening the antioxidative processes in plants (Noctor et al. 2011; Gill et al. 2013). Reestablishing functional reduced form of glutathione and preserving the adequate redox homeostasis proved to be crucial (Tsai et al. 2005).

Studies using *Arabidopsis* mutants that are defective in GR have demonstrated that the ability to maintain high cellular GSH/GSSG ratios is an essential determinant of plant organ growth and vigor (Diaz-Vivancos et al. 2015). Although loss of GR1 did not affect the amount of GSH, the whole-cell GSSG level was increased in *gr1* mutants compared to wild-type leaves. Despite the reduction of GR activity

by 65 % and a lower GSH/GSSG ratio, *gr1* deletion mutants are able to undergo normal plant development (Marty et al. 2009). Using redox-sensitive green fluorescent protein (GRX1-roGFP2), it was shown that E_{GSH} in *gr1* mutants is significantly shifted toward more oxidizing conditions. Dynamic reduction of GSSG formed during induced oxidative stress in *gr1* mutants was still possible, probably due to the NADPH-dependent thioredoxin (TRX) system which could work as a backup system for GR1 (Marty et al. 2009). Furthermore, using *gr1* and *catalase2 gr1* (*cat2 gr1*) double mutants, Mhamdi et al. (2010) found that GR1 plays a specific role in H_2O_2 responses, when intracellular H_2O_2 production is increased, particularly during stresses. However, their results related to the analysis of transcriptomic pattern suggested that GR–GRX systems may function grandly independently of TRXs in oxidative stress signaling, at least at the level of transcript abundance (Mhamdi et al. 2010).

In contrast to GR1, GR2 is essential for the development of *Arabidopsis* plants. GR2 deletion mutants produced a lethal phenotype and showed growth arrest at the stage of early embryo development (Tzafrir et al. 2004; Diaz-Vivancos et al. 2015). A GR2 mutant called *miao* (a non-lethal mutation of *GR2* gene), which shows reduced GR activity, about half of that in the wild-type plants, displayed strong root apical meristem defects and root growth inhibition (Yu et al. 2013). Yu et al. (2013) demonstrated that glutathione oxidation is responsible for the root phenotype observed in *miao*. Furthermore, according to the result of the experiments with GR2 RNAi plants, GR2 is an important regulator of leaf senescence and is vital in maintaining both the function of the acceptor side of PSII and in the repair of photodamaged PSII by preventing the accumulation of H_2O_2 (Ding et al. 2016a, b).

Using tobacco chloroplastic GR RNAi plants, it was also shown that the capacity for the regeneration of glutathione by GR is crucial in the protection against oxidative stress, by maintaining the ascorbate pool and the ascorbate redox state (Ding et al. 2009). In antisense transgenic tomato lines, the decrease of glutathione reductase activity affected glutathione regeneration and consequently influenced ascorbate regeneration and total ascorbate content, which resulted in greater accumulation of H_2O_2 and an enhanced sensitivity to chilling stress (Shu et al. 2011). A number of recent studies have been implemented on the role of GR transgenes in conferring abiotic stress tolerance (Rao and Reddy 2008; Gill et al. 2013; Achary et al. 2015; Rajeevkumar et al. 2015). Briefly, in several GR overexpressing transgenic plants elevated GR activity resulted in increased stress tolerance to a variety of abiotic stresses.

It was reported that GR also undergoes redox interconversions, depending upon the substrate availability. The oxidized form of the enzyme is more stable than the reduced form, ensuring the effective work of the enzyme even under adverse conditions. GR was not affected by peroxynitrite ($ONOO^-$) or *S*-nitrosoglutathione (GSNO), suggesting the existence of a mechanism to conserve redox status by maintaining the level of reduced GSH (Begara-Morales et al. 2015). The oxidized form of GR also showed tolerance to divalent metal ions such as Zn^{2+}, Cu^{2+} and Fe^{2+} (Rao and Reddy 2008).

7.4 GSTs are a Large and Even Broadening Family of Proteins Which Comprise Highly Heterogenic Enzymes with Diverse Structure and Function

GSTs constitute a very ancient protein superfamily that is thought to have evolved from a thioredoxin-like ancestor, in response to the development of oxidative stress (Martin 1995; Sheehan et al. 2001; Mohsenzadeh et al. 2011). While mammalian GSTs have been extensively investigated and classified according to generally agreed criteria, a number of novel GST classes were first identified in non-mammalian sources. At the beginning, plant GSTs were allocated to theta class, since those GSTs show generally lower amino acid sequence identities than other classes in animals (Sheehan et al. 2001). The revealed diversity of the GST sequences and gene organization (Droog et al. 1995), their differently induced activity (Mozer et al. 1983) and variable safener specificity (Irzyk and Fuerst 1993) led to the division of the plant GSTs into three distinct groups (I, II and III), which was intended to be an analogous classification system to the mammalian GSTs [alpha, mu, pi, theta, sigma system (Droog et al. 1995; Marrs 1996; Dixon et al. 1998a, b)]. The three groups of GSTs included herbicide-detoxifying enzymes differing in the number of the contained exons in their genes: Type I included GSTs with three exons; Type II contained 10 exons; and Type III consists of GSTs containing two exons. An extension of this classification into Type IV was then proposed for a group of *Arabidopsis thaliana* GSTs which are closely related to the mammalian theta class (Dixon et al. 1998b).

As in 1999 dozens of plant GST sequences were reported, the previous classification became inappropriate. Edwards et al. (2000) adopted the widely used Greek-letter designations of non-plant GSTs and amended the nomenclature of plant GSTs, including the following new classes: the two plant-specific classes are termed phi (GSTF, previously Type I) and tau (GSTU, previously Type III), the theta class includes enzymes previously designated as Type IV, while the zeta class includes those previously classified as Type II. In addition, Dixon et al. (2002) reported two further plant-specific classes in *Arabidopsis* (lambda, L; dehydroascorbate reductase, DHAR). This research group later identified further genes related to GSTs: a predicted protein that most closely resembles the bacterial-like tetrachlorohydroquinone dehalogenase (TCHQD), and a microsomal GST with glutathione-dependent activity (Dixon et al. 2002; Edwards and Dixon 2005). According to sequence similarity, immunological cross-reactivity, substrate specificity and genome organization, soluble GSTs have been further grouped into different species-independent classes, and some of them are specific to kingdoms or phyla (Munyampundu et al. 2016). Based on structural similarities, the γ-subunit of the eukaryotic translation elongation factor 1B (EF1Bγ) has also come to be regarded as a member of the GST family, as RIRT and APNG amino acid motifs are found at a position similar to the active site signature in other GSTs (Jeppesen et al. 2003; Oakley 2005; Lan et al. 2009; Lallement et al. 2014). Among the proteins involved in the mitochondrial preprotein import on the outer membrane,

metaxin was characterized to play a role in the import of mitochondrial precursor proteins and is likely to have a function in the assembly of β-barrel proteins into the outer membrane. Besides the transmembrane domain, two putative conserved GST domains were identified in plant metaxin, which revealed the possible relations to GSTs (Lister et al. 2007; Carrie et al. 2010). Nine additional groups of plant GSTs were identified by Liu et al. (2013) in the genome of the moss *Physcomitrella patens*, these sequences belonged to the bacterial and fungal Ure2p. Eight out of the nine *P. patens* GSTs contained both a GST and a hemerythrin domain and were grouped into one distinct clade and were named as "hemerythrin class" (GSTH) GSTs. The remaining one sequence was grouped as a distinct clade named the "iota class" GST (GSTI) (Liu et al. 2013). Furthermore, microsomal prostaglandin E synthase type 2 (mPEGS-2) enzymes with several representative in planta were defined as a new member of GSTs by Lallement et al. (2014), regarding the GSH-dependent activity, structure similarity and the presence of the two GST domains. The classification and potential function of plant GSTs are summarized in Table 7.1.

GSTs usually form dimers between subunits showing relative high sequence identity. Molecular recognition at the subunit interface is absolutely class specific, since dimerization involves subunits only from the same GST class. Such in-class heterodimers were reported in maize and in tomato (Sommer and Boger 1999; Kilili et al. 2004). Each subunit contains an active site that appears as a cleft along the domain interface, which is located in the inner part of the dimeric molecule (Dirr et al. 1994; Cummins et al. 2011). According to the crystal structures of plant GSTs, each monomer consists of two distinct domains, connected with a linker region (Armstrong 1993). Domain I, located in the N-terminal region, contains the hydrophilic G site for binding the physiological substrate glutathione and includes the following structural elements: $\beta_1\alpha_1\beta_2(\alpha_2)\beta_3\beta_4\alpha_3$. The substrate-binding H site consists of entirely α-helical structures and is located in domain II, in the C-terminal region of the subunits (Armstrong 1997; Cummins et al. 2011). Between the two domains of the subunits, a hypervariable linker region is located. Comparing the numerous described GSTs structures, some features were found to be variable among the classes: α_2/loop region, the linker region, the bend in α_4- and α_6-helix and the length of α_9-helix (Cummins et al. 2011).

Regarding the several functions under control conditions and in abiotic stress responses, plant GSTs seem to have a high degree of functional overlap and variability, both within and between classes. Among the classes, the plant-specific tau and phi GSTs are the most abundant. They are involved mainly in the xenobiotic metabolism, which may be related to the high affinity toward a broad spectrum of harmful compounds, including xenobiotics and endogenous stress metabolites, e.g., lipid peroxides and reactive aldehydes, and may result in high tolerance to abiotic stresses (Gallé et al. 2009; Dixon and Edwards 2010a; Liu et al. 2013; Csiszár et al. 2014; Horváth et al. 2015a; b) (Fig. 7.1). According to their steady-state kinetic constants against standard xenobiotic substrates, such as 1-chloro-2,4-dinitrobenzene (CDNB), 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole, or 1,2-dichloro-4-nitrobenzene, the contribution of these GST groups to GSH diminution is also variable. In poplar for example,

Table 7.1 Classification, abbreviations and main characteristics of plant GSTs

GST class	Short form	Greek letter	Catalytic amino acid	Main activities	Known functions briefly
Phi	F	φ	Ser	Glutathionylation GSH-dependent peroxidase	Detoxification of cytotoxic compounds Reduction of organic hydroperoxides
Tau	U	τ	Ser	Glutathionylation GSH-dependent peroxidase	Detoxification of cytotoxic compounds Reduction of organic hydroperoxides
Theta	T	θ	Ser	GSH-dependent peroxidase	Reduction of organic hydroperoxides
Zeta	Z	ζ	Ser	GSH-dependent isomerase Dehalogenation	Tyr catabolism Xenobiotic recycling
Lambda	L	λ	Cys	GSH-dependent thiol transferase, deglutathionylation	Maintenance of flavonoid pool
Hemerythrin	H		Cys	Deglutathionylation	
Iota	I	ι	Cys	Deglutathionylation	
Ure2p	ure2p		APNG motif	Glutathionylation, GSH-dependent peroxidase	
Glutathionyl hydroquinone reductase	GHR		Cys	GSH-dependent thiol transferase, deglutathionylation	Reduction of oxidized quinones
Elongation factor 1B gamma subunit	EF1Bγ		RIRT motif	Glutathionylation	
Dehydroascorbate reductase	DHAR		Cys	GSH-dependent thiol transferase	Maintenance of reduced ascorbate pool Regulation of ASC and GSH redox states
Tetrachlorohydroquinone dehalogenase	TCHQD		Ser	Dehalogenation	Detoxification of chlorinated compounds, xenobiotic detoxification
Metaxin	M		Cys	GSH-dependent thiol transferase	Import of mitochondrial precursor proteins
Microsomal prostaglandin E synthase type 2	mPEGS-2		Cys	GSH-dependent thiol transferase	Isomerization of prostaglandin H_2

See text for more details

among the members of tau group GSTs, there is an almost two thousand-fold difference in the $(K_{cat}/K_m)^{GSH}$ constant for CDNB. Regarding the results originated from wheat, poplar, *Arabidopsis*, tomato and *Physcomitrella patens,* the differences in the steady-state kinetic constants within the group members of phi and tau classes seem to be higher than the interclass variability (Kilili et al. 2004; Lan et al. 2009; Dixon et al. 2010; Liu et al. 2013). Anyway, their catalytic activity may reduce the GSH pool by using it as a co-substrate. Theta and zeta GSTs have very restricted glutathione-conjugating activities toward xenobiotics. Theta GSTs are involved also in oxidative stress metabolism, but they are particularly efficient as glutathione peroxidases, using GSH to reduce organic hydroperoxides to alcohols (Basantani and Srivastava 2007; Dixon and Edwards 2010a). Zeta GSTs participate in Tyr catabolism and have GSH-dependent isomerase activity (Edwards and Dixon 2005). Among the other groups, EF1Bγ1 exhibited GSH-conjugating activity, though, according to the kinetic properties, it was suggested to have a small influence on the GSH pool (Liu et al. 2013). Interestingly, plants contain also glutathionyl hydroquinone reductase (GHR) class GSTs. The fungal and bacterial GHRs can reduce glutathionylated (chlorinated) (hydro)quinones with K_{cat} around 10^3 s^{-1} and K_{cat}/K_m up to 10^6 M^{-1} s^{-1} (Xun et al. 2010; Lallement et al. 2014). Although there are some contrasting data in the literature, most of the GHRs are able to catalyze the deglutathionylation of GSH-conjugated oxidized quinones and are regarded to have a central role in the regulation of the quinone redox state, possibly preventing their toxicity, either naturally present or found as environmental pollutants. Moreover, it is also possible that GHRs have protein substrates and have similar roles to that of glutaredoxins (Lam et al. 2012; Lallement et al. 2014).

GSTs belonging to GHR, DHAR, GSTL, mPEGS-2 and metaxin classes contain a Cys residue in their G site, in the place of the usual serine residue (Table 7.1), which radically changes enzyme properties (Dixon and Edwards 2010b; Lallement et al. 2014). The so-called Cys-GSTs have no detectable GSH-conjugating activity toward standard xenobiotic GST substrates, but have glutathione-dependent thiol transferase activity and catalyze redox reactions, even deglutathionylation, by performing nucleophilic attacks on various GSH-conjugated substrates. In Cys-GSTs, the catalytic Cys becomes glutathionylated. The regeneration of these glutathionylated GST forms requires a GSH molecule, which is oxidized to glutathione disulfide. The formed GSSG will be reduced back by GR, at the expense of NADPH. DHARs and GSTLs are also able to perform deglutathionylation reactions, similarly to glutaredoxins, but instead of oxidized proteins, their targets are usually metabolites, especially oxidized antioxidants (Lallement et al. 2014). GSTLs appear to be unique to terrestrial plants. The stress-inducible wheat TaGSTL1 was found to selectively bind flavonols, e.g., quercetin and taxifolin, and the binding proved to be dependent upon *S*-glutathionylation of the active site Cys. The GSTLs may have a role in maintaining the flavonoid pool under stress conditions (Dixon and Edwards 2010b). DHARs reduce dehydroascorbate (DHA) to ascorbate, while oxidizing GSH to GSSG (Dixon et al. 2002). Hence, DHARs are of great importance in the regulation of cellular ASC redox state, e.g., DHAR-overexpressing tobacco plants were found to have an increased ASC redox

state in guard cells (Chen and Gallie 2004; Zhang et al. 2015). Noshi et al. (2016) reported that DHAR3 regulates both ascorbate and glutathione redox states in *Arabidopsis* during high light stress. It was proved that the *Arabidopsis* DHAR2 has redox-active Cys which is regulated through a sulfenylation and *S*-glutathionylation. Waszczak et al. (2014) demonstrated that the DHAR activity was maintained in the presence of 1 mM GSH after adding 1 mM H_2O_2, so in this case this posttranslational protein modification protected the protein against oxidative damages. Most Cys-GSTs have a single Cys in their active site, but a few isoforms have an additional one in the active site motif, such as *Arabidopsis* DHAR3. The AtDHAR3 was shown to form an intramolecular disulfide upon GSSG treatment (Dixon et al. 2002), but is not known whether it constitutes an intermediate step of the catalysis or is part of the protective mechanism that prevents overoxidation of the catalytic Cys. A possible connection with thioredoxins was also suggested, because TRXs may be involved in their reduction (Lallement et al. 2014). Interestingly, the DHAR proteins with CPFC active site motif show cytoplasmatic localization, while others with CPFS active site (containing single Cys) are fairly all cytosolic. A mutation of the cytosolic AtDHAR, which reduces the apoplastic DHA, resulted in increased ozone sensitivity, indicating the importance of this system in oxidative stress responses (Yoshida et al. 2006). Furthermore, overexpression of cytosolic DHAR in rice, potato and tobacco plants enhanced tolerance to herbicide, salt, drought and ozone stresses (Eltayeb et al. 2006, 2011; Kim et al. 2014). The overexpression of DHAR with GR, or GST with GR improved protection against both methyl viologen and chilling. The combination of transgenes increased the regeneration of reduced ascorbate and glutathione and participated in a more rapid scavenging of superoxide radicals and hydrogen peroxide, prior to their interaction with target molecules (Le Martret et al. 2011).

7.5 Glutathione Peroxidase may be a Link Between Glutathione and Thioredoxin Systems

Glutathione peroxidase enzymes (EC 1.11.1.9 and EC 1.11.1.12) are widespread among eukaryotic organisms and can also be found in prokaryotes (Brenot et al. 2004). The GPX gene family has an uncertain origin and does not follow a linear evolutionary history, but they could be visualized in three main clusters with polyphyletic origins: vertebrates and invertebrates GPX group, bacteria and fungi GPX group and plants GPX group (Margis et al. 2008). Detailed phylogenetic analysis of thiol peroxidases, including peroxiredoxins, and plant, fungi and animal GPXs revealed that plant GPXs are more closely related to human GPX4 than to fungal GPXs (Margis et al. 2008).

Glutathione peroxidase was discovered in 1957, as an enzyme in erythrocytes, which is capable of protecting hemoglobin from oxidative breakdown, by catalyzing the oxidation of the reduced glutathione by H_2O_2 (Mills 1957). The enzyme

was discovered a second time as a mitochondrial protein, which has a role in the mitochondrial swelling–contraction cycle (Green and O'brien 1970). Analysis of these proteins revealed that selenocysteines in their active site are responsible for the peroxidase activity (Flohe et al. 1973). More than a decade later, the presence of GPX activity was reported for the first time in fungi (Galiazzo et al. 1987). Later, it has been proven that not all GPX contain selenocysteine, because purified enzyme from *Euglena* showed selenium-independent glutathione peroxidase activity (Overbaugh and Fall 1985). The presence of GPXs in higher plants was debated until the evidence of GPX activity in different cultured plant cells (Drotar et al. 1985).

At molecular level, plant GPX genes are closely related to animal phospholipid hydroperoxide glutathione peroxidases (Margis et al. 2008). The majority of animal GPXs are selenium-containing enzymes, while plant GPXs are selenium-independent monomeric proteins, containing cysteine in their catalytic site, therefore showing lower activity than their animal counterparts (Eshdat et al. 1997). Three conserved cysteine residues can be found in GPX proteins, which are essential for the enzymatic process, but only two cysteines form an intramolecular disulfide bridge and take part in the regeneration catalytic cycle (Navrot et al. 2006).

The plant GPXs are classified as the fifth group of peroxiredoxins, because it was revealed that they use the thioredoxin—rather than the glutathione—system during the reduction of H_2O_2 and lipid peroxides (Iqbal et al. 2006). It was reported that some of them have both glutathione peroxidase and thioredoxin peroxidase functions, but the thioredoxin-regenerating system is much more efficient than the glutathione system. It was determined that the catalytic efficiencies of tomato and sunflower GPXs are higher in the presence of thioredoxin (K_m 2.2 and 1.5 μM, V_{max} 263.2 and 243.9 nmol min^{-1} mg^{-1}) than in the presence of glutathione (K_m 9300 and 4900 μM, V_{max} 48.8 and 46.7 nmol min^{-1} mg^{-1}) (Herbette et al. 2002). Other experiments indicated that plant GPXs use only TRX, but not GSH in the reduction of hydroperoxides (Jung et al. 2002; Iqbal et al. 2006; Herbette et al. 2007). GPXs were suggested to be a putative link between the glutathione-based and the thioredoxin-based system (Jung et al. 2002; Milla et al. 2003); furthermore, a redundancy was established between glutathione and TRX systems (Noctor et al. 2011).

Some plant GPXs are more efficient in reducing lipid peroxides (K_m 12.1–128 μM, V_{max} 15.8-57.5 nmol min^{-1} mg^{-1}) than H_2O_2 (K_m was not detectable) (Herbette et al. 2002; Navrot et al. 2006), which can imply their role in the protection of membranes. Plant GPXs are considered to be relatively weak peroxidase scavengers, compared with other GPXs or APX (Eshdat et al. 1997). It is suggested that they work in tandem with peroxiredoxins to detoxify peroxides and are also involved in the regulation of the redox homeostasis by maintaining the thiol/disulfide or NADPH/$NADP^+$ balance (Ozyigit et al. 2016). Although most of plant GPXs have higher affinity toward the thioredoxin substrate (Herbette et al. 2002; Iqbal et al. 2006), the reduced level of GSH (in *rml1* mutants) affects the transcript level of *GPX*s (Schnaubelt et al. 2015).

During abiotic stresses, GPXs can play multiple roles. Their function shows some overlaps with peroxiredoxins and GSTs in the maintenance of H_2O_2 homeostasis by the elimination of H_2O_2 and organic hydroperoxides. Therefore, GPXs are part of the scavenging enzymes against oxidative damages, and their significant role was emphasized especially in the protection of membranes (Herbette et al. 2002). *GPX* transcription levels were increased by oxidative stress and various abiotic stress conditions (Li et al. 2000; Yang et al. 2005; Navrot et al. 2006; Diao et al. 2014; Sugimoto et al. 2014; Bela et al. 2015). GPXs functioned as a cytoprotector in yeast and tobacco, preventing Bax-, H_2O_2-, heat- and salt stress-induced cell death (Chen et al. 2004). It was reported that overexpression of GPX genes in tomato led to higher tolerance against abiotic stress factors (Herbette et al. 2011). Transgenic tomato plants harboring a mammalian *GPx* maintained a significantly higher photosynthesis rate and fructose-1,6-bisphosphatase activity under chilling stress, and the role of modified levels of the ASC and GSH redox couples was suggested in the sustained viability (Herbette et al. 2005). It was suggested that GPXs are involved in redox signaling mechanisms too (Foyer and Noctor 2005).

7.6 GSH-Related Enzymes and the Redox-Dependent Signaling

Current concepts emphasize a redox-regulated aspect of cell homeostasis, involving individual ROS, reactive nitrogen and sulfur species, ROS-producing enzymes, antioxidants, their oxidized/reduced forms, and their role in mediating plant responses to abiotic stress conditions (Potters et al. 2010). One of the main signaling functions of ROS relays on reversible redox-based posttranslational modifications of thiols which may result in modified physical conformations or biochemical activities (Foyer and Noctor 2005). Oxidative modifications of proteins involve disulfides, *S*-glutathionylation, *S*-nitrosylation and oxidation of a Cys thiol group to a sulfenic acid (Cys-SOH), which can act as a regulatory switch in several signal transduction pathways (Ma et al. 2007). (See also Chapter 19. Begara-Morales et al. "Protein *S*-Nitrosylation and *S*-Glutathionylation as regulators of redox homeostasis during abiotic stress response".) Sulfenic acid formation is reversible, but they can react rapidly with other thiols to form intra- or intermolecular disulfides for example by *S*-glutathionylation, unless they are stabilized into the protein environment. These mechanisms protect the sulfenic acids against overoxidation to sulfinic (SO_2H) or sulfonic (SO_3H) acid (Waszczak et al. 2014), as it was indicated by the redox-active Cys-containing proteins. Similarly to GR and AtDHAR2, the oxidized form was also active in the case of γ-ECS (GSH1) enzyme (Hicks et al. 2007), whereas a general example for proteins where the reduced form is active is the NPR1 (non-expressor of pathogenesis-related genes 1 protein)

(Mou et al. 2003); however, the connection between the redox state and glutathione-related enzymes is more complex.

It is well established that these enzymes may affect the GSH and ascorbate levels and their reduced states (Foyer and Noctor 2005; Chang et al. 2009). Besides the various functions of GSH discussed earlier, a broad spectrum of molecular investigations revealed that the amount of glutathione and the redox potential has a large influence on the regulation of biological functions under normal and stress conditions (Foyer and Noctor 2005; Szalai et al. 2009; Zagorchev et al. 2013). There have been several studies about the effect of exogenous GSH on the activity and/or transcript amount glutathione-related enzymes; however, its effect is rather controversial (Szalai et al. 2009). The depletion of GSH in *Arabidopsis* mutants is associated with redox state modifications causing vast changes in gene expression (Dubreuil-Maurizi et al. 2011; Schnaubelt et al. 2015; Kumar et al. 2015). Transcript profiling analysis of *Arabidopsis* mutants with impaired GSH biosynthesis or after application of the GSH synthesis inhibitor buthionine sulfoximine (BSO) revealed different abundance of several hundreds of transcripts, particularly those that encode transcription factors and proteins involved in hormone-dependent regulation of plant growth and development. Among the genes regulated by low GSH were GSTs, DHARs, GPXs, h-type TRXs and GRXs (Schnaubelt et al. 2015). There was a big variability in different isoenzymes coding genes. For example, the expression of *AtGSTF11*, *AtGSTF14*, *AtGSTU20* and *AtGPX1*, *AtGPX7* were downregulated, while that of the *AtGSTF6*, *AtGSTF16*, *AtGSTU1*, *AtGSTU4*, *AtGSTU24*, *AtGSTU25* and *AtGPX6* were upregulated in *rml1* shoots, compared with the wild-type plants (Schnaubelt et al. 2015). Several GST and GPX proteins were reported to interact with other proteins; hence, they are considered to have signaling functions (Delaunay et al. 2002; Dixon and Edwards 2010a). For example, Loyall et al. (2000) suggested the involvement of a GST in signal transduction pathways which may be in connection with the redox state of the cells. Overexpression of an UV light-inducible GST gene increased the tolerance of the transgenic plants to UV radiation, and the involvement of GST, glutathione and the oxidative status of the cells in early events of a UV light-dependent signal transduction pathway to chalcone synthase was indicated (Liu and Li 2002). Miao et al. (2006) reported that *Arabidopsis* GPX3 (AtGPX3) can interact with ABI1 and ABI2 (abscisic acid-insensitive) phosphatases, leading to stomatal closure via activation of cation channels. AtGPX3 can also interact in yeast two-hybrid system with the transcriptional regulator CEO1 protein, which can control several genes involved in plant stress responses (Miao et al. 2006). It has been demonstrated that the redox state of the *Arabidopsis* AtGPX3 is regulated by H_2O_2 and indicated that this GPX is a redox transducer in abscisic acid (ABA) and drought stress signaling. GPXs were suggested to function even as ROS or redox sensors (Milla et al. 2003), but this effect has been not yet confirmed. However, putative signaling functions were assigned to AtGPX8, as it was supposed that this isoenzyme may take part in the redox modification of nuclear proteins (Gaber et al. 2012).

7.7 Concluding Remark

Although the pivotal role of glutathione- and GSH-related enzymes has been known for several decades and they were investigated intensively together with other antioxidants and multiple stress physiological parameters in a wide range of plant species, varieties or cultivars under broad spectrum of abiotic stresses, our knowledge about this complex mechanism is far not satisfactory. The recent literature supplied increasing evidence of the GSH and the redox state being involved in the transcriptional control of various genes in physiological and developmental processes. In the light of the recent findings, GSH-related enzymes are not simply enzymatic compounds of the ascorbate–glutathione cycle, but have a more general function in the maintenance of the redox state of cellular compounds, and at least some of them may be a key participant in redox signaling. It became clear that significant functional overlaps exist not only between the different GSH-related enzymes, but also with other redox systems, as glutaredoxins, thioredoxins and peroxiredoxins. Besides revealing other endogenous substrates, recognition of further protein–protein interactions may provide significantly new information about them in the future.

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